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Developmental Effects of the ToxCast™ Phase I and II Chemicals in *Caenorhabditis elegans* and Corresponding Responses in Zebrafish, Rats, and Rabbits

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Running title: Effect of ToxCast™ chemicals on *C. elegans*

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Abstract

Background: Modern toxicology is shifting from an observational to a more mechanistic science. As part of this shift, high-throughput toxicity assays are being developed using alternative, non-mammalian species to prioritize chemicals and develop prediction models of human toxicity.

Methods: The nematode *Caenorhabditis elegans* (*C. elegans*) was used to screen the EPA's ToxCast™ Phase I and II libraries, containing 292 and 676 chemicals respectively, for chemicals leading to decreased larval development and growth. Chemical toxicity was evaluated using three parameters: a biologically defined effect size threshold, half-maximal activity concentration (AC_{50}), and lowest effective concentration (LEC).

Results: Across both the Phase I and II libraries, 62% of the chemicals were classified as active up to 200 μM in the *C. elegans* assay. Chemical activities and potencies in *C. elegans* were compared to those from two zebrafish embryonic development toxicity studies and developmental toxicity data for rats and rabbits. Concordance of chemical activity was higher between *C. elegans* and one zebrafish assay across Phase I chemicals (79%) than with a second zebrafish assay (59%). Using *C. elegans* or zebrafish to predict rat or rabbit developmental toxicity resulted in balanced accuracies, the average value of the sensitivity and specificity for an assay, ranging from 45 – 53%, slightly lower than the concordance between rat and rabbit (58%).

Conclusions: Here we present an assay that quantitatively and reliably describes the effects of chemical toxicants on *C. elegans* growth and development. We found significant overlap in the activity of chemicals in the ToxCast™ libraries between *C. elegans* and zebrafish developmental screens. Incorporating *C. elegans* toxicological assays as part of a battery of *in vitro* and *in vivo*

assays provides additional information for the development of models predicting a chemical's potential toxicity to humans.

Introduction

The U.S. National Toxicology Program (NTP) is charged with providing current scientific information to regulatory agencies and the general public on the potential human health risks of environmental toxicants. Little to no toxicity information is available for thousands of chemicals currently in use. To address this paucity of information, the Tox21 community was established through a memorandum of understanding between the NTP, the U.S. Environmental Protection Agency (EPA), and the National Institutes of Health Chemical Genomics Center, now the National Center for Advancing Translational Sciences (NCATS) (Collins et al. 2008). Tox21 is using high-throughput *in vitro* screening and *in vivo* alternative animal model testing to identify mechanisms of toxicity, prioritize chemicals for additional *in vivo* toxicity testing, and develop predictive models of human toxicological responses. As part of that effort, the EPA-National Center for Computational Toxicology (NCCT) ToxCast™ program uses batteries of *in vitro* assays in an attempt to prioritize thousands of chemicals for further toxicological testing and develop prediction models for human toxicity (Dix et al. 2007).

The ToxCast™ Phase I library contains 292 unique chemicals, comprised mainly of pesticide active ingredients (Judson et al. 2010). These chemicals are relatively well-characterized by traditional mammalian toxicity tests: data from rat and rabbit developmental toxicity tests are available for 251 and 234 of these 292 chemicals, respectively, in the EPA's Toxicity Reference Database (ToxRefDB) (Knudsen et al. 2009). The Phase II library contains 676 unique chemicals that included nine chemicals from the Phase I library as well as additional 14replicates that function as internal tests for reproducibility. While the chemical space is much broader for Phase II than Phase I, including failed pharmaceuticals, food additives, and industrial products, many of these chemicals have not been tested in traditional mammalian assays. Human clinical

data, however, are available for some of the chemical classes, such as cosmetics and failed pharmaceuticals, allowing for direct linkage to human health effects (http://www.epa.gov/ncct/toxcast/files/ToxCast%20Chemical%20Summary%2014Dec2010.pdf).

Unlike high-throughput *in vitro* assays, which can rapidly provide information on large numbers of chemicals at low cost, whole animal models are more labor intensive, time consuming, and costly, and thus are used to test smaller numbers of chemicals (Collins et al. 2008). Nevertheless, animal models offer certain advantages over cell-based testing models. For example, chemical effects on multiple, interacting cell types and can be used to monitor a variety of phenotypic endpoints impacted by chemical exposures (e.g., overall reproductive effects). Thus, whole-animal assays allow for the examination of complex phenotypes, often involving multiple mechanisms, and may better represent human exposure situations.

Animal species with short developmental periods and phenotypes that can be measured using automated processes are particularly useful in rapidly estimating chemical effects on whole organism development. The nematode *Caenorhabditis elegans* has been shown to be amenable to this process (Benson et al. 2014; Boyd et al. 2010b; Leung et al. 2011). *C. elegans* is also widely used as a model for human diseases including age-associated neurodegenerative diseases, genetic diseases, and metabolic disorders (Aitlhadj et al. 2011; Kaletta and Hengartner 2006). Previous work using *C. elegans* as a toxicological model found predictive relationships between locomotion and reproduction endpoints in *C. elegans* and lethality in rodents (Boyd et al. 2010a; Cole et al. 2004; Melstrom and Williams 2007; Williams and Dusenbery 1988).

The *C. elegans* larval growth and development assay presented in this publication provides an indication of a chemical's effects on nematode growth and development. *C. elegans* growth, like

many lower organisms, is not a continuous process but occurs through four distinct molts with differing sizes (Byerly et al. 1976). This assay quantifies the size of individual nematodes as optical density or extinction (EXT using a COPAS Biosort flow cytometer (Pulak 2006), after 48-h continuous exposures to chemicals beginning with L1 larvae. In untreated *C. elegans*, the population at 48 h will develop to the L4 stage, such that there is a direct relationship between size and EXT. In comparison, exposed animals generally range in size and developmental stage from L1 to L4, depending on the severity of growth inhibition invoked by chemical exposures. Chemical exposures were limited to 48 h to avoid the production of a second generation of offspring, which would complicate data analysis. Under highly toxic conditions, nematodes decrease in size or die during the 48 h exposure (Boyd et al. 2009; Smith et al. 2009).

The goal of the current study was to determine the inhibition of *C. elegans* larval growth after exposures to the ToxCast^{TM} Phase I and II chemicals. A subset of the Phase I chemicals, with known but variable growth inhibitory potencies, was first used to test the reliability and reproducibility of this assay. Optical absorption measurements were then linked with visually observed developmental stages to define a biologically relevant "effect size threshold" that was used to assess chemical activity. Because the *C. elegans* assay coincided with larval development, the *C. elegans* hazard classifications were compared to several other *in vivo* assays for which exposures occurred during development of the animals: zebrafish embryonic development toxicity assays (Padilla et al. 2012; Truong et al. 2014) and rat and rabbit developmental toxicity data (Sipes et al. 2011a).

Methods

Nematode culture

The Bristol N2 (wild-type) strain of *C. elegans* was obtained from the *Caenorhabditis* Genetic Center and maintained at 20°C on K-agar plates (2% bacto-agar, 0.25% bacto-peptone, 51 mM sodium chloride, 32 mM potassium chloride, 13 μM cholesterol) seeded with *E. coli* OP50 as a food source (Brenner 1974; Williams and Dusenbery 1988). Age-synchronized adult nematodes were prepared using alkaline-hypochlorite treatment, as previously described (Khanna et al. 1997).

Chemicals

The chemicals in the ToxCast™ Phase I and II libraries (http://www. epa. gov/NCCT/toxcast/chemicals.html) were provided by the U.S. EPA in 100% DMSO, typically at concentrations of 20 mM. As 1% DMSO did not affect *C. elegans* growth (Supplemental Material, Figure S1), chemicals were diluted with complete K-medium (51 mM sodium chloride, 32 mM potassium chloride, 3 mM calcium chloride, 3 mM magnesium sulfate, 13 μM cholesterol) to a maximum concentration of 200 μM. Exposures to 4% DMSO were sub-lethal and almost completely inhibit nematode growth (Supplemental Material, Figure S1). Thus, 4% DMSO was used as the positive control for all experiments.

C. elegans growth assay

Growth assays were modified from Boyd et al. (Boyd et al. 2009) and employed the COPAS Biosort flow sorting system (Pulak 2006) (Union Biometrica Inc.). The Biosort was used to dispense 50 age-synchronized L1 larvae into each well of a 96-well plate containing complete K-medium, varying concentrations of the test chemical (0.5, 1, 5, 10, 50, 100, and 200 μM), 1%

DMSO (final concentration), and killed OP50 *E. coli*. Nematodes were exposed to chemicals at 48 h at 20°C, at which time untreated nematodes reached the L4 to young adult stage (Smith et al. 2009). The Biosort was then used to measure the EXT of individual nematodes at one time immediately following 48 hchemical exposures and the values converted to natural log(EXT) for analyses. Biosort measurements of extraneous material such as detritus, bacteria clumps, or precipitates were filtered from the data using a growth model, as previously described (Boyd et al. 2010b; Smith et al. 2009).

The screens of Phase I and Phase II libraries were initiated three years apart (May 2008 for Phase I and May 2011 for Phase II), and the plate design was slightly altered during this time. In both screens, each 96 well plate consisted of a single concentration of eight chemicals, as well as the negative control (1% DMSO) and positive control (4% DMSO). Additional concentrations were tested on separate 96 well plates. For Phase I, chemicals were loaded within rows with four wells per treatment group and rinse wells between each treatment well. For Phase II, chemicals were loaded within columns with six wells per treatment group followed by two rinse wells. Rinse wells contained 1% DMSO and were placed between treatment groups to rinse the aspiration tool and avoid carryover of animals between adjacent treatment groups. Plate adjustments were made by subtracting the mean nematode size of the plate negative controls (i.e., 1% DMSO only treated nematodes) of each plate, which had average log(EXT) of 5.665, with an arbitrary values of six added for display purposes to allow a decreasing response as toxicity increases with no effect on the analysis. Subsequent analyses (lowest effective concentrations (LECs) calculations, Hill function estimates, Z scores, etc.) were performed using the mean size of the nematodes within an individual well after 48 h chemical exposures.

Classifying chemical activity by C. elegans larval development

To determine the performance characteristics of the *C. elegans* growth assay, ten replicate plates containing eight chemicals each, with a wide range of growth inhibitory effects on *C. elegans* (parathion, dichlorvos, diazinon, lindane, methyl-isothiocyanate, carbaryl, isoxaben, and ethephon) were examined. Each plate contained four wells of each chemical at 200 μM, and negative (1% DMSO) and positive (4% DMSO) controls. The EXT values were directly linked to *C. elegans* developmental stage by examining all wells containing nematodes by microscope to determine the larval stages. Mean sizes of all nematodes (log(EXT)) within wells containing only a single larval stage were plotted against larval stage number only for Figure 1. For these analyses, 837 wells contained at least one nematode and 432 of these wells contained larvae from only one developmental stage. Wells with mixed larvae were used in all subsequent analyses. The minimum log(EXT) value from any negative control or treatment wells containing only L4s or young adults was used as an effect size threshold. In addition, for each replicate plate, Z-factors were calculated as described by Zhang et al. (1999) for the 1% DMSO vehicle control samples compared with the 4% DMSO positive control samples, as well as with parathion and dichlorvos, the two most active *C. elegans* toxicants. The Z-factor provides a measure of assay quality by taking into account both the dynamic range and data reliability within one number (Zhang et al. 1999).

Active chemicals in Phase I were identified using both the effect size threshold and weighted t-test, which compared log(EXT) well means from treated groups to the negative controls on the same plate. Both the t-test and the effect size threshold were used to estimate two sets of LECs for all Phase I chemicals. The(log(EXT)) values of nematodes after 48 h exposures for each chemical were fit to a Hill function, using weighted regression with a genetic algorithm (Mullen

et al. 2011) with wells having ten or more nematodes. For five chemical exposures at the highest concentration (200 μM), less than ten nematodes were sampled per well. By microscope, all of the nematodes were observed to be dead. Because these chemicals were also active at 100 μM with ten or more living nematodes present per treatment well, the 200 μM data was not necessary and excluded from toxicity estimation. The following constraints were used to prevent the generation of parameter estimates outside of the feasible concentration region during the fitting of the Hill function: the top asymptote was constrained to be in [0, 10], the exponent in [0, 25], the AC_{50} estimate in [0, 1000], and the lower asymptote in [3.135, 10].

Interspecies comparisons

The *C. elegans* larval development results from the Phase I and Phase II chemical libraries were compared to the Zebrafish^{T} embryonic developmental assay using published LEC values (Truong et al. 2014). Results for the Phase I chemicals from the *C. elegans* larval development assay were additionally compared to the Zebrafish^{P} embryonic developmental assay using published AC_{50} estimates (Padilla et al. 2012). Two developmental summary endpoints for rats and rabbits from the ToxRef database (http://www.epa.gov/ncct/toxrefdb/) (Knudsen et al. 2009), "DEV_rat_Developmental" and "DEV_rabbbit_Developmental" were also compared using chemicals from the Phase I library (Sipes et al. 2011b). The outcomes given for these summary statistics are minimum LEC values over the included endpoints.

Outcomes among the four species were compared using performance metrics for classification of compounds as active or inactive (sensitivity, specificity, and balanced accuracy) as well as Kendall's tau as a concordance measure. Sensitivity is the proportion of all active compounds identified as active; specificity is the proportion of all inactive compounds identified as inactive.

Because a test may be very good in either sensitivity or specificity but not the other, balanced accuracy (the average of sensitivity and specificity) is also calculated. Predicted classifications as active or inactive were compared across the combined chemicals with results for all species, as well as within 9 chemical classes identified within the Phase I library (Judson et al. 2010). As repeated observations on the replicate chemicals in the Phase I dataset were not available for mammalian or zebrafish data, comparisons between species were analyzed using averaged *C. elegans* results.

Results

C. elegans growth assay performance

Eight chemicals from the Phase I library with a range of growth inhibitory effects were selected to evaluate data quality and calibrate the range of biological effects for this assay. Mean Z-factors and standard deviations were calculated for these eight chemicals and the positive control (4% DMSO). L1 and L2 stages as observed by microscopic examination were observed for the positive control, parathion, dichlorvos and diazinon . Lindane treatments resulted in all L3 larvae for at least one replicate. Of the remaining four chemicals, methyl-isothiocyanate and carbaryl showed mixtures of L3s and L4s, and isoxaben and ethephon were similar to the negative controls: just L4s and young adults. Because Z-factors compare the means and standard deviations of highly toxic compounds and negative controls (Zhang et al. 1999), only the positive control and two most toxic chemicals (parathion and dichlorvos) were used to calculate Z-factors. Mean Z-factors (± SD) relative to negative controls based on 10 replicate plates for the parathion and dichlorvos were 0.779 ± 0.068 and 0.859 ± 0.034, respectively, and 0.698 ± 0.175 for the positive control (See Supplemental Material, Tables S1, S2 and S3 for the Z-statistics

data for each of the 10 replicate plates). A Z-factor between 0.5 and 1.0 indicates a clear separation between treated and untreated groups and is considered an 'excellent assay' (Zhang et al. 1999). As evidenced by mean Z-factors and their small standard deviations, the *C. elegans* growth assay displayed a high degree of consistency between replicate measurements with a clear separation between affected and unaffected groups.

To link measured EXT values directly to specific *C. elegans* stages of development, exposed nematodes were visually examined to determine the larval stages present. A comparison between mean sizes (log(EXT)) of nematodes within each well in a treatment group containing only a single larval stage and the visually observed developmental stage is presented in Figure 1. Following a 48 h incubation, the mean log(EXT) of L4 larvae and young adults were larger than 5.665, while L1 - L3 larvae were all smaller than 5.138. The lowest mean log(EXT) of 3.135 corresponded to L1 larvae, indicating very little growth during the 48 h exposure. Because untreated animals were L4s at the end of the exposure period, an effect size threshold was defined such that exposed nematodes with mean log(EXT) less than 5.665 were considered different from controls.

Classifying chemical activity on C. elegans larval development

To classify the chemical activity of the compounds in the Phase I library at the highest concentration tested (200 μM), two methods were examined: a weighted t-test and the effect size threshold. For the t-test, mean log(EXT) values of exposed nematodes were weighted by the number of nematodes and then compared to those from vehicle controls within the same plate. Using this method, 232 or 79.5% of unique Phase I chemicals were identified as active at an overall $p < 0.05$ level (Bonferroni-corrected $p < 0.05/292 = 0.000171232$) (Figure 2 and

Supplemental Material, Excel Table S1). Using the effect size threshold of mean log(EXT) < 5.665 identified 200 chemicals as active that were also identified by the t-test, as well as seven additional chemicals; 32 chemicals were identified active only by the t-test. Additionally, 53 compounds were inactive in both methods. Because the effect size threshold reflects the biological significance of a chemicals growth inhibitory effect, it was used to classify compound activity for the remaining comparisons.

All chemicals from both the Phase 1 and Phase 2 libraries were screened at seven concentrations: 0.5, 1, 5, 10, 50, 100 and 200 μM. Two classical toxicological metrics were used to define potency: lowest effective concentrations (LECs) and half-maximal active concentrations (AC_{50}s) estimated from fitting the Hill function (Supplemental Material, Excel Table S2; Hill plots for each of the tested chemicals are available upon request from the authors). LECs were defined as the lowest concentration at which the mean log(EXT) of the exposed nematodes was less than the effect size threshold and remained below this threshold for subsequent, higher concentrations (Table 1).

Interspecies comparisons of toxicity: ToxCast™ Phase I

Comparison to zebrafish development

The *C. elegans* results for the Phase I chemical library were compared to those from two zebrafish embryo developmental assays referred to as Zebrafish^{P} (Padilla et al. 2012) and Zebrafish^{T} (Truong et al. 2014). Zebrafish^{P} estimated AC_{50}s and AC_{10}s using a composite deformity score after chemical exposures at 1 nM to 80 μM, while Zebrafish^{T} estimated LECs across 18 endpoints including mortality after exposure to chemicals at 6.4 nM to 64 μM. The minimum LEC calculated from all 18 zebrafish embryonic development endpoints was used for

comparisons to *C. elegans* data. Of 292 unique chemicals, there was agreement between all three assays for 152 compounds; 119 active; 33 inactive (Figure 3) for a concordance of 0.52. The two zebrafish assays agreed on 191 chemicals (145 active; 46 inactive) with a concordance of 0.65, while Zebrafish^{P} results agreed with the *C. elegans* results on 232 chemicals (182 active; 50 inactive) with a concordance of 0.79 and Zebrafish^{T} results agreed with the *C. elegans* results on 173 chemicals (131 active; 42 inactive) with a concordance of 0.59. The potency rank of the Phase I chemicals were also compared between *C. elegans* and the two zebrafish assays. Comparing 122 AC_{50}s with estimates less than the maximum tested concentration between *C. elegans* and Zebrafish^{P} (Supplemental Material, Excel Table S2), a non-significant correlation of 0.078 was estimated by Kendall's tau ($p = 0.40$). Comparing LEC values between Zebrafish^{T} and *C. elegans*, a slight, but significant, correlation was estimated (Kendall's tau = 0.108; $p = 0.021$).

Comparison to mammalian development

The U.S. EPA's Toxicity Reference Database (ToxRefDB) (Martin et al. 2009) contains summary statistics consisting of minimum LECs for 27 developmental outcomes for rats and 26 developmental outcomes for rabbits exposed to most of the Phase I chemicals (DEV_rat_Developmental and DEV_rabbit_Developmental, respectively) (Sipes et al. 2011a). Composite LECs for the rabbit and rat developmental endpoints were available for 234 and 251 chemicals, respectively. A chemical was classified as inactive for these outcomes if it was tested, but no LEC was reported. The rat and rabbit composite LECs were compared to LECs and AC_{50}s from *C. elegans* and the two zebrafish embryonic development assays. For the 200 chemicals tested in all species, the percent active chemicals in the Phase I library were 71% for C. *elegans*, 75% for Zebrafish^{P}, 61% for Zebrafish^{T}, 43% for rabbits, and 59% for rats. Balanced

accuracy estimates (the average of sensitivity and specificity) for predicting rat and rabbit developmental toxicity based on *C. elegans* assays were 52% and 53%, respectively, compared with corresponding estimates for the two zebrafish assays of 51–52% and 45-50% (Table 2). *C. elegans* assays were the most sensitive for rabbit toxicity (74% compared with 60-68%) and ZebrafishP assays were the most sensitive for rat toxicity (76% compared with 61-74%). The specificity of *C. elegans* assays for predicting rabbit and rat toxicity was 30% and 32%, respectively, while corresponding values for the Zebrafish assays were 21-39% and 38-40%. The concordance between rat and rabbit development was 58%, with 59/200 active and 56/200 inactive in both.

Comparison by chemical class

The activities of the Phase I chemicals within previously described chemical classes (Judson et al. 2010) were assessed in *C. elegans*, zebrafish, rat, and rabbit development (Table 3 and Table 4). The most active chemical class across species was conazoles, with the lowest number of active chemicals observed in rabbit. Amides, anilides, and organophosphates had a higher percentage of active chemicals in nematodes and zebrafish than in rats and rabbits. Overall, Zebrafish^{P} had the highest proportion of active chemicals, followed by *C. elegans* and then Zebrafish^{T}, while rabbit had the lowest proportion of actives.

The concordance between *C. elegans* growth and the two zebrafish embryonic development assays within Phase I chemical classes is presented in Table 3. As observed for all of the Phase I chemicals, the *C. elegans* growth results agree well with Zebrafish^{P} across most of the chemical categories. However, although similar numbers of urea chemicals were active in both assays, the concordance was only 38%: *C. elegans* indicated 5 active and 3 inactive, Zebrafish^{P} identified 6

active and 2 inactive, but only 3 of 8 chemicals were classified the same by both assays (Table 3). The concordance between *C. elegans* and Zebrafish^{T} was highest for conazoles, carbamates and pyrethroids, and was otherwise 50% or less. The concordance between the two zebrafish assays was less than 50% for the phenoxy and urea chemical classes.

Finally, the *C. elegans* and two zebrafish assay results were used to predict activity in rat and rabbit development within chemical classes using balanced accuracy estimates (Table 4). Overall, zebrafish and *C. elegans* prediction of mammalian outcomes were similar within most chemical classes. The balanced accuracies for prediction of rabbit development using *C. elegans* growth were highest for anilide (0.81), amide (0.76) and urea (0.75), while all of the balanced accuracies for prediction of rat from *C. elegans* were ≤ 0.70. For Zebrafish^{P}, balanced accuracies for rat were highest for phenoxy (0.75), pyridine (0.75) and carbamate (0.70) classes, and for rabbit for amide (0.72) and carbamate (0.71). Balanced accuracies for Zebrafish^{P} were lowest for urea compounds (0.30 in rats and 0.17 in rabbits), but highest for Zebrafish^{T} (0.90 in rats and 0.75 in rabbits). The combined sensitivity and specificity of *C. elegans* assays for urea compounds was low for rats (0.30) and comparable to Zebrafish^{T} for rabbits (0.75).

Combined ToxCast™ Phase I & II

Activity in C. elegans larval growth & development assay

In Figure 4, the 959 unique chemicals from the combined Phase I and Phase II libraries are clustered using the mean log(EXT) for the *C. elegans* assay at all concentrations tested. Overall, the number of active chemicals and intensity of effect monotonically increased with concentration. The 50 chemicals with the greatest effect on growth at the highest concentration tested (200 μM) were comprised mainly of pesticides and included several organophosphates

(chlorpyrifos, chlorpyrifos oxon, isazofos, coumaphos, O-Ethyl O-(p-nitrophenyl) phenylphosphonothioate (EPN)), organotins (triphenyltin hydroxide, tributyltin chloride, and tributyltin methacrylate), avermectins (abamectin, emamectin benzoate, and milbemectin), and organochlorines (DDD, DDT, DDE, and dicofol). Nineteen of the 50 chemicals were also active at the lowest concentration tested (0.5 μM) (Supplemental Material, Excel Table S2 and Supplemental Material, Table S4); these chemicals listed by increasing mean log(EXT) at 0.5 μM are: emamectin benzoate, abamectin, fentin, milbemectin, pyridaben, isazofos, quinoxyfen, tebufenpyrad, chlorpyrifos oxon, fenpyroximate, coumaphos, methylene bis(thiocyanate), molinate, fenamiphos, pyriproxyfen, oxyfluorfen, parathion, methoxychlor and dicofol.

Replicate Analysis

Replicate chemicals were included by the ToxCast program in each library to monitor assay performance (Table 5). The Phase I library included four chemicals replicated twice (3-iodo-2-propynylbutylcarbamate (IPBC), dibutyl phthalate (DBP), S-Ethyl dipropylthiocarbamate (EPTC), and fenoxaprop-ethyl) and two that were replicated three times (bensulide and diclofop-methyl) and Phase II library contained seven chemicals from the Phase I library replicated three times (allethrin, azoxystrobin, bisphenol A, oryzalin, perfluorooctane sulfonic acid (PFOS), triadimenol, and triclosan) and two additional chemicals from Phase I that were replicated six times (clorophene and mancozeb). Chemicals with LECs or AC_{50}s of 200 μM or less were classified as active, while those with no LEC and AC_{50} were inactive. Most of the chemicals were classified as active in all replicate samples except EPTC, which was inactive in both replicates. In two cases the chemicals did not agree across all replicates: mancozeb was inactive when tested with the Phase I library, but was active in all six replicates within the Phase II library; and triadimenol was active in two replicates and inactive in the other two. In both cases,

the chemicals were weakly active even at 200 μM, as evidenced by mean sizes (represented by log(EXT) at 200 μM) near the size effect threshold of 5.665. In contrast with classification as active or inactive, LEC and AC_{50} values varied among the replicate samples.

Comparison to zebrafish development

Combined results for Phase I and II chemicals were available for *C. elegans* and Zebrafish^{T}. Of the 959 unique chemicals, the two assays agreed for 560 chemicals (363 active and 197 inactive) for a concordance of 0.58. Zebrafish^{T} classified 167 chemnicals as active that were inactive in the *C. elegans* assay; and 232 chemicals were active based on the *C. elegans* assay but inactive based on Zebrafish^{T}. Kendall's tau was used to compare LECs by rank and was estimated to be 0.102 ($p = 9.7 \times 10^{-5}$). Using only the 603 compounds where Zebrafish^{T} mortality occurred at a higher concentration than the first teratogenic effect or did not occur at all (Truong et al. 2014), the nematodes and zebrafish agree on 314 compounds (117 active and 197 inactive) for a concordance of 0.52.

Discussion

The current study presents a high-throughput whole animal screen using the nematode *C. elegans*. *C. elegans* and other *in vivo* animal models offer many benefits over cell-based models in the prediction of human toxicological responses. However, the ability of any animal model, from nematodes to mammals, to respond in a manner similar to humans is limited by how well the organism and toxicological assays replicate human exposure conditions (stage of development, route of exposure, etc.) and cellular, biochemical and molecular responses. Like all *in vivo* models, *C. elegans* contains many processes similar to higher organisms (Shaye and Greenwald 2011). Likewise, it is deficient in others. Although *C. elegans* cannot replicate all of

the processes necessary to predict the effects of all compounds in humans, its level of homology with humans is sufficient to include it with other *in vivo* models in predictive toxicology and the development of adverse outcome pathways. A thorough review of conserved toxicity pathways can be found in the 2000 National Research Council report (National Research Council (U.S.). Committee on Developmental Toxicology. 2000).

The *C. elegans* automated assay uses COPAS Biosort flow cytometry to screen for the effects of chemicals on C. *elegans* larval growth and development. The results presented in this paper show that the C. *elegans* growth assay produced excellent Z-scores with values for the positive control and two active chemicals between 0.5 and 1 (Zhang et al. 1999) and consistency of responses across 10 replicates indicating that the assay produces responses to chemicals that are highly reproducible and distinguishable from untreated controls. The assay also produced reliable hazard identification at the highest concentration tested across replicate chemicals within the ToxCast™ Phase I and II libraries (Table 5).

Two methods were applied to classify chemical activity: a statistical t-test and a newly defined effect size threshold (Figure 1). The statistical t-test determined the difference between exposed and control groups, incorporating variability of the samples and providing p-values. The low variability within the samples, however, led to a number of compounds being classified as having statistically significant effects on growth, even though little difference in size was measured. With relatively few compounds inducing growth inhibition classified as inactive by the t-test, the effect size threshold was used for the remainder of the analysis (Figure 2). Thus, if the mean log(EXT) of exposed nematodes was less than the effect size threshold, the chemical was classified as active.

Nineteen chemicals were classified as most active by hierarchical clustering of the effect size (Figure 4) and were active at the lowest concentration tested (0.5 μM) (Supplemental Material, Table S4 and Excel Table S2). Not surprisingly several avermectins, which are pesticides primarily used to control parasitic nematodes, mites, fleas, and other insects, were classified as actives. Two of the avermectins most toxic to *C. elegans*, emamectin benzoate and abamectin, were potent inhibitors of development in both Zebrafish^{P} and Zebrafish^{T}, and have also been shown to be potent inhibitors of spontaneous movement in zebrafish embryos indicating potential developmental neurotoxic effects (Raftery et al. 2014). A number of other compounds, which are known or suspected developmental neurotoxicants in a number of *in vitro* and *in vivo* models (Crofton et al. 2011; Grandjean and Landrigan 2014), were also among the most toxic chemicals to *C. elegans* in this study, including the organophosphate chlorpyrifos and its metabolite chlorpyrifos oxon; the organochlorine DDT and its metabolites; two tributyltin compounds and triphenlytin; and several polyaromatic hydrocarbons (PAHs) (Supplemental Material, Table S4).

Two different zebrafish embryonic development assays were compared to the *C. elegans* results: the Zebrafish^{P} assay (Padilla et al. 2012), with results for only the Phase I chemicals, and the Zebrafish^{T} assay (Truong et al. 2014) with results for Phase I and II chemicals. We note that while both the *C. elegans* and the Zebrafish^{P} assays determined activity on severity of treatment effect, the Zebrafish^{T} assay determined activity on the basis of incidence of treatment effect. Other major differences in experimental design between the two studies included the presence or absence of the acellular chorion, repeat versus static exposures, and manual versus automated morphometric analyses. Overall, the *C. elegans* larval development assay was found to have excellent agreement with Zebrafish^{P} embryo development with a concordance of almost 80% for

the Phase I chemicals, while the concordance with the Zebrafish^{T} assay was lower at 59% for Phase I and 58% for Phase I and II, respectively.

Both the C. *elegans* and zebrafish assays describe developmental effects of chemical exposures, therefore responses in these species were compared to developmental effects indices for rats and rabbits in ToxRefDB for the 200 Phase I chemicals tested in all four species. By using a combination of a suite of developmental outcomes (Sipes et al. 2011a), the numbers of active and inactive chemicals, as identified by these two indices, were reasonably well balanced. A clear pattern of chemical activity prediction, however, did not emerge. While the Zebrafish^{P} and *C. elegans* assays did have high concordance, neither predicted classification of activity in either rabbits or rats (combined average sensitivity and specificity ~ 50%, Table 2). While the balanced accuracies for these assays were similar to those from Zebrafish^{T}, the concordance was much lower. Again, this discrepancy is likely due to the measurement of incidence in the Zebrafish^{T} studies vs. the measurement of severity of response in the rat and rabbit studies. The rats and rabbits did provide some information for each other, but with lower concordance than might have been expected (~ 58%).

Interestingly, the poor performance of the two predictor species (*C. elegans* and zebrafish) was not uniform across chemical classes within the Phase I library (Table 3 and Table 4). When predictions were evaluated within chemical classes (Table 4), balanced accuracy ranged from a high of 81% (*C. elegans* predicting rabbit endpoints for anilide compounds) to a low of 17% (Zebrafish^{P} predicting rabbit toxicity for urea compounds). When Phase I and II chemical activity at each concentration are grouped using Hierarchical clustering, chemicals within chemical classes appear to be clustering together (Figure 4; Supplemental Material, Table S4).

Taken together, the large disparity in predictive powers between chemical classes and clustering of activity suggest that QSAR methods could play a large role in the eventual predictive battery of assays.

Throughout the results presented in this paper, the estimation or prediction of potency was found to be less reliable than identification or concordance of chemical activity. Table 5 shows response estimates (i.e., mean size or log(EXT)) at the high concentration to be very consistent across replicates, while the AC_{50} estimates vary to a much greater extent. In cross-species comparisons, although the concordance of *C. elegans* active predictions of Zebrafish^{P} were quite good at 0.79, no significant correlation was found between chemical potencies (Kendall's tau coefficient 0.078; $p = 0.40$).

Conclusions

Here we present an assay that quantitatively and reliably describes the effects of chemical toxicants on *C. elegans* growth and development. We found substantial overlap in the activity of chemicals in the ToxCast™ Phase I library in the Zebrafish^{P} and *C. elegans* developmental screens, but lower concordance between *C. elegans* and the Zebrafish^{T} developmental screens for the combined Phase I and II libraries. Prediction of mammalian effects from *C. elegans* or zebrafish responses was poor across the Phase I library, but was higher within certain chemical classes-assay combinations. Incorporating other *C. elegans* toxicological assays, such as feeding (Boyd et al. 2007) and reproduction (Boyd et al. 2010a), could provide additional insights into the specificity of endpoints and yield further information adding to the overall utility of *C. elegans* as an alternative toxicological model. We propose using *C. elegans* assays as part of a battery of toxicity tests and analytical methods including *in silico* modeling and prediction, cell-

free and cell-based *in vitro* assays, alternative toxicological model organisms such as zebrafish and daphnia, traditional toxicological model organisms such as rodents and rabbits, and relevant human data including clinical and epidemiological observations.

References

Aitlhadj L, Avila DS, Benedetto A, Aschner M, Sturzenbaum SR. 2011. Environmental exposure, obesity, and Parkinson's disease: lessons from fat and old worms. Environ Health Perspect 119:20-28.

Benson JA, Cummings EE, O'Reilly LP, Lee MH, Pak SC. 2014. A high-content assay for identifying small molecules that reprogram *C. elegans* germ cell fate. Methods 68:529-535.

Boyd WA, McBride SJ, Freedman JH. 2007. Effects of genetic mutations and chemical exposures on *Caenorhabditis elegans* feeding: evaluation of a novel, high-throughput screening assay. PLoS ONE 2:e1259.

Boyd WA, Smith MV, Kissling GE, Rice JR, Snyder DW, Portier CJ, et al. 2009. Application of a mathematical model to describe the effects of chlorpyrifos on *Caenorhabditis elegans* development. PLoS One 4:e7024.

Boyd WA, McBride SJ, Rice JR, Snyder DW, Freedman JH. 2010a. A high-throughput method for assessing chemical toxicity using a *Caenorhabditis elegans* reproduction assay. Toxicol Appl Pharmacol 245:153-159.

Boyd WA, Smith MV, Kissling GE, Freedman JH. 2010b. Medium- and high-throughput screening of neurotoxicants using *C. elegans*. Neurotoxicol Teratol 32:68-73.

Brenner S. 1974. The genetics of *Caenorhabditis elegans*. Genetics 77:71-94.

Byerly L, Cassada RC, Russell RL. 1976. The life cycle of the nematode *Caenorhabditis elegans*. I. Wild-type growth and reproduction. Dev Biol 51:23-33.

Cole RD, Anderson GL, Williams PL. 2004. The nematode *Caenorhabditis elegans* as a model of organophosphate-induced mammalian neurotoxicity. Toxicol Appl Pharmacol 194:248-256.

Collins FS, Gray GM, Bucher JR. 2008. Toxicology. Transforming environmental health protection. Science 319:906-907.

Crofton KM, Mundy WR, Lein PJ, Bal-Price A, Coecke S, Seiler AE, et al. 2011. Developmental neurotoxicity testing: recommendations for developing alternative methods for the screening and prioritization of chemicals. Altex 28:9-15.

Dix DJ, Houck KA, Martin MT, Richard AM, Setzer RW, Kavlock RJ. 2007. The ToxCast program for prioritizing toxicity testing of environmental chemicals. Toxicol Sci 95:5-12.

Grandjean P, Landrigan PJ. 2014. Neurobehavioural effects of developmental toxicity. The Lancet Neurology 13:330-338.

Judson RS, Houck KA, Kavlock RJ, Knudsen TB, Martin MT, Mortensen HM, et al. 2010. *In vitro* screening of environmental chemicals for targeted testing prioritization: the ToxCast project. Environ Health Perspect 118:485-492.

Kaletta T, Hengartner MO. 2006. Finding function in novel targets: *C. elegans* as a model organism. Nat Rev Drug Discov 5:387-398.

Khanna N, Cressman CP, 3rd, Tatara CP, Williams PL. 1997. Tolerance of the nematode *Caenorhabditis elegans* to pH, salinity, and hardness in aquatic media. Arch Environ Contam Toxicol 32:110-114.

Knudsen TB, Martin MT, Kavlock RJ, Judson RS, Dix DJ, Singh AV. 2009. Profiling the activity of environmental chemicals in prenatal developmental toxicity studies using the U.S. EPA's ToxRefDB. Reprod Toxicol 28:209-219.

Leung CK, Deonarine A, Strange K, Choe KP. 2011. High-throughput screening and biosensing with fluorescent *C. elegans* strains. J Vis Exp 19:2745.

Martin MT, Judson RS, Reif DM, Kavlock RJ, Dix DJ. 2009. Profiling chemicals based on chronic toxicity results from the U.S. EPA ToxRef Database. Environ Health Perspect 117:392-399.

Melstrom PC, Williams PL. 2007. Reversible AChE inhibitors in *C. elegans* vs. rats, mice. Biochem Biophys Res Commun 357:200-205.

Mullen KM, Ardia D, Gil DL, Windover D, Cline J. 2011. DEoptim: An R package for global optimization by differential evolution. J Stat Softw 40:1-26.

National Research Council (U.S.). Committee on Developmental Toxicology. 2000. Scientific frontiers in developmental toxicology and risk assessment. Washington, DC:National Academy Press.

Padilla S, Corum D, Padnos B, Hunter DL, Beam A, Houck KA, et al. 2012. Zebrafish developmental screening of the ToxCast Phase I chemical library. Reprod Toxicol 33:174-187.

Pulak R. 2006. Techniques for analysis, sorting, and dispensing of *C. elegans* on the COPAS flow-sorting system. Methods Mol Biol 351:275-286.

Raftery TD, Isales GM, Yozzo KL, Volz DC. 2014. High-content screening assay for identification of chemicals impacting spontaneous activity in zebrafish embryos. Environ Sci Technol 48:804-810.

Shaye DD, Greenwald I. 2011. OrthoList: a compendium of *C. elegans* genes with human orthologs. PLoS One 6:e20085.

Sipes NS, Martin MT, Reif DM, Kleinstreuer NC, Judson RS, Singh AV, et al. 2011a. Predictive models of prenatal developmental toxicity from ToxCast high-throughput screening data. Toxicol Sci 124:109-127.

Sipes NS, Padilla S, Knudsen TB. 2011b. Zebrafish: as an integrative model for twenty-first century toxicity testing. Birth defects research Part C, Embryo today : reviews 93:256-267.

Smith MV, Boyd WA, Kissling GE, Rice JR, Snyder DW, Portier CJ, et al. 2009. A discrete time model for the analysis of medium-throughput *C. elegans* growth data. PLoS One 4:e7018.

Truong L, Mandrell D, Mandrell R, Simonich M, Tanguay RL. 2014. A rapid throughput approach identifies cognitive deficits in adult zebrafish from developmental exposure to polybrominated flame retardants. Neurotoxicology 43:134-142.

Williams PL, Dusenbery DB. 1988. Using the nematode *Caenorhabditis elegans* to predict mammalian acute lethality to metallic salts. Toxicol Ind Health 4:469-478.

Zhang JH, Chung TD, Oldenburg KR. 1999. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J Biomol Screen 4:67-73.

Table 1. Number (percent) of Phase I and Phase II chemicals with LECs at tested concentrations

Library	Chemical Concentration (μM)							
	0.5	**1**	**5**	**10**	**50**	**100**	**200**	**>200[b]**
Phase I	19 (6.5%)	5 (1.7%)	10 (3.4%)	12 (4.1%)	46 (15.8%)	25 (8.6%)	89 (30.5%)	86 (29.4%)
Phase II	16 (2.4%)	9 (1.3%)	35 (5.2%)	35 (5.2%)	86 (12.7%)	51 (7.5%)	164 (24.3%)	280 (41.4%)
Total	35	14	45	47	132	76	253	366
Cumulative Total	**35**	**49**	**94**	**141**	**273**	**349**	**602**	**968[a]**

[a]Nine chemicals are replicated in the Phase I and II libraries, so 959 unique chemicals across both libraries.

[b] "LEC >200" indicates a compound that my affect nematode growth above the tested concentrations 0.5 – 200 μM. These compounds may also be inactive at any concentration.

Table 2. Accuracy of *C. elegans* or zebrafish embryogenesis toxicity data for predicting developmental outcomes in rabbits and rats

Predicting species	Predicted species[a]	
	Rabbit	**Rat**
C. elegans		
BA	52.3%	52.7%
Sensitivity	74.1%	73.7%
Specificity	30.4%	31.7%
Zebrafish^{P}		
BA	44.6%	52.2%
Sensitivity	68.2%	76.3%
Specificity	20.9%	28.0%
Zebrafish^{T}		
BA	49.6%	50.6%
Sensitivity	60.0%	61.0%
Specificity	39.1%	40.2%

BA = Balanced Accuracy = average of sensitivity and specificity

Data were available across all species for 200 unique chemicals. Zebrafish^{P} is from Padilla, *et al*. 2012 (Padilla et al. 2012) and Zebrafish^{T} is from Truong, *et al*. 2014 (Truong et al. 2014)

[a]The species listed in each row was used to predict the outcome of the species across columns.

Table 3. Proportion of chemicals classified as active and concordance between assays among groups of Phase I chemicals.

Chemical Class[a] (number of chemicals)	Proportion active[b]			Concordance[c]		
	C. elegans	Zebrafish^{P}	Zebrafish^{T}	*C. elegans* and Zebrafish^{P}	*C. elegans* and Zebrafish^{T}	Zebrafish^{P} and Zebrafish^{T}
Amide (24)	0.58	0.75	0.75	0.75	0.50	0.75
Anilide (14)	0.64	0.79	0.86	0.86	0.50	0.64
Carbamate (15)	0.53	0.80	0.67	0.60	0.73	0.73
Conazole (18)	1.00	1.00	0.89	1.00	0.89	0.89
Organophosphate (35)	0.80	0.86	0.57	0.83	0.49	0.54
Phenoxy (12)	0.67	0.92	0.33	0.75	0.33	0.42
Pyrethroid (12)	0.92	1.00	0.67	0.92	0.58	0.67
Pyridine (10)	0.70	0.60	0.40	0.90	0.50	0.60
Urea (8)	0.63	0.75	0.63	0.38	0.50	0.38

[a]Chemical classes were derived from Judson et al. (Judson et al. 2010)

[b]Chemical activity is based on the specific assays for Zebrafish^{P} (Padilla et al. 2012), Zebrafish^{T} (Truong et al. 2014) and *C. elegans* (this publication)

[c]Concordance is defined as the proportion of chemicals with the same classification

Table 4. Balanced accuracy[a] of *C. elegans*, Zebrafish[P], and Zebrafish[T] assays for predicting developmental outcomes in rabbits and rats according to chemical class

Chemical class	Rats[c]					Rabbits[c]				
	n	% active	*C. elegans*	Zebrafish[P]	Zebrafish[T]	n	% active	*C. elegans*	Zebrafish[P]	Zebrafish[T]
Amide	21	0.48	0.62	0.63	0.54	22	0.36	0.76	0.72	0.42
Anilide	14	0.50	0.57	0.57	0.50	14	0.43	0.81	0.69	0.33
Carbamate	14	0.71	0.50	0.70	0.43	14	0.50	0.64	0.71	0.71
Conazole	16	1.00	All active[b]	All active[b]	2 inactive[b]	16	0.69	0.50	0.50	0.41
Organophosphate	25	0.32	0.43	0.53	0.36	25	0.24	0.50	0.58	0.60
Phenoxy	8	0.75	0.50	0.75	0.75	11	0.27	0.52	0.33	0.31
Pyrethroid	12	0.50	0.42	0.50	0.33	10	0.40	0.38	0.50	0.33
Pyridine	7	0.43	0.63	0.75	0.42	6	0.50	0.33	0.50	1.00
Urea	6	0.83	0.30	0.30	0.90	5	0.60	0.75	0.17	0.75

[a]The sum of the average sensitivity and specificity

[b]Unable to calculate balanced accuracy due to the absence of sufficient negative results.

[c] Data for rats and rabbits were obtained from ToxRef database (http://www.epa.gov/ncct/toxrefdb/) (Knudsen et al. 2009)

Table 5. Replicate concordance among chemicals in the Phase I and II libraries

Chemical	Phase	Log(EXT) at 200 µM	LEC	AC_{50}	Hazard[a]
Allethrin	I	5.37	50	NC[b]	active
	II	5.39	100	NC	active
	II	4.93	50	NC	active
	II	5.22	200	NC	active
Azoxystrobin	I	5.51	200	195.8	active
	II	5.60	200	NC	active
	II	5.43	200	196.5	active
	II	5.44	50	NC	active
Bensulide	I	3.71	50	16.3	active
	I	3.83	50	13.7	active
	I	3.49	100	79.8	active
Bisphenol A	I	5.37	200	NC	active
	II	5.57	200	NC	active
	II	5.38	200	NC	active
	II	5.52	200	NC	active
Clorophene	I	3.61	200	68.8	active
	II	3.87	10	160.6	active
	II	3.83	50	57.6	active
	II	3.65	50	84.9	active
	II	3.92	0.5	80.9	active
	II	3.79	50	113.6	active
	II	3.91	50	39.4	active
Dibutyl phthalate	I	5.58	200	NC	active
	I	5.26	50	21.3	active
Diclofop-methyl	I	4.92	200	179.0	active
	I	4.46	50	179.3	active
	I	4.47	50	56.2	active
EPTC	I	6.02		NC	inactive
	I	5.70		NC	inactive
Fenoxaprop-ethyl	I	5.01	100	76.7	active
	I	5.36	50	46.0	active
IPBC	I	3.00	200	138.7	active
	I	3.34	100	74.3	active
Mancozeb	I	5.75		NC	inactive
	II	5.35	200	NC	active
	II	5.37	200	NC	active
	II	5.24	100	NC	active

	II	5.47	0.5	124.1	active
	II	5.24	100	NC	active
	II	5.29	200	NC	active
Oryzalin	I	3.97	50	136.3	active
	II	4.95	50	19.6	active
	II	4.72	10	49.9	active
	II	4.57	10	NC	active
PFOS	I	3.66	200	177.3	active
	II	3.06	5	18.5	active
	II	3.22	0.5	13.5	active
	II	3.39	5	6.1	active
Triadimenol	I	4.99	200	189.4	active
	II	5.63	200	NC	active
	II	5.94		NC	inactive
	II	5.79		NC	inactive
Triclosan	I	3.98	50	109.6	active
	II	3.83	10	69.1	active
	II	4.06	50	43.2	active
	II	4.15	10	26.3	active

[a]Chemicals were classified as "active" if they had an LEC or $AC_{50} \leq 200$ μM otherwise they were classified as "inactive."

[b]NC, could not be calculated.

Figure Legends

Figure 1. Association between *C. elegans* size and developmental stage. Nematode developmental stages (L1 larva - Adult) were determined after direct observation by microscope and then size characteristics (EXT) were measured using the COPAS Biosort. The mean log(EXT) of the nematodes in each well for a treatment group, which contained nematodes at a single larval stage are presented. The log(EXT) of L4s and young adults were all greater than 5.665 (dotted line); nematodes that had not developed to the L3 stage were all less than 5.138 (dashed line). Each point represents the mean size (log (EXT)) of the nematodes in an individual well.

Figure 2. Comparison between t-test and effect size threshold. The histogram presents the number of chemicals in each size class (mean log(EXT)). Dark gray indicates the number of inactive compounds in each size class according to the weighted t-test, while light gray indicates the number of compounds determined to be active in each size class. The two vertical lines indicate the maximum log(EXT) for nematodes ≤ L3 (5.138), and the minimum log(EXT) (5.665) for L4 and young adult nematodes (see Figure 1). Chemicals between the vertical lines had weighted mean Log(EXT) values consistent with a mixture of L3s and L4s.

Figure 3. Concordance between *C. elegans* larval development and zebrafish embryonic development assays for ToxCast™ Phase I chemical activity. Venn diagram illustrating the concordance between the effects of chemicals on *C. elegans* development and two zebrafish development assays: Zebrafish^{P} (Padilla et al. 2012)and Zebrafish^{T} (Truong et al. 2014).

Figure 4. Hierarchical clustering of chemical activity on *C. elegans* development. *upper panel*; activity of 959 unique chemicals from ToxCast™ Phase I and II libraries clustered according to mean log(EXT). *lower panel*; activity and chemical names of the 50 chemicals with the greatest effect on *C. elegans* growth. Lists and descriptions of chemicals in the lower panel are presented in Supplemental Material, Table S4. *legend;* Blue corresponds to inactive chemicals with responses similar to controls, while yellow to red indicates decreasing nematode size with increasing toxicity. The histogram illustrates the size distribution of matched negative controls.

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Figure 1

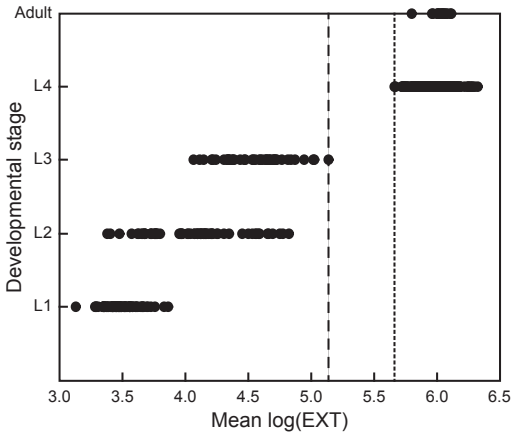

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Figure 2

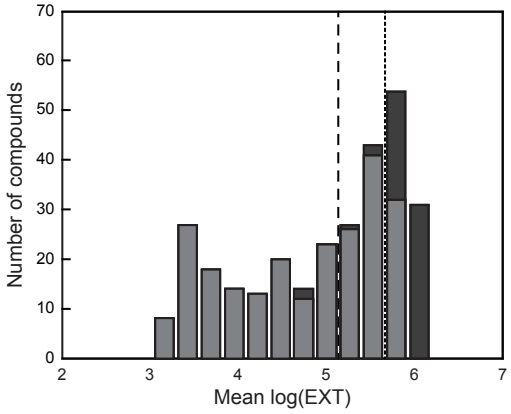

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Figure 3

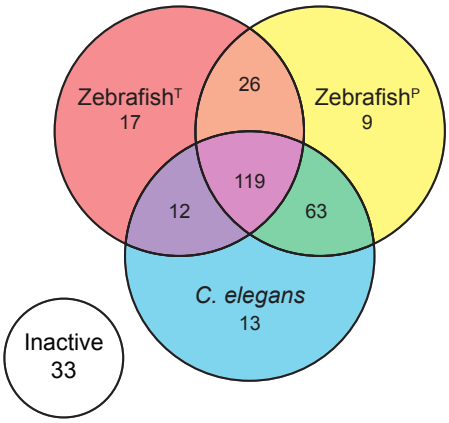

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Figure 4